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(54) Title: DELIVERY OF BIOACTIVE COMPOUNDS TO AN ORGANISM

(57) Abstract

Disclosed herein is a method of delivering a bioactive compound to an organism that involves growing individual cells *in vitro* under conditions that allow the formation of an organized tissue, at least a subset of the cells containing a foreign DNA sequence which mediates the production of the bioactive compound; and implanting to organized tissue into the organism, whereby the bioactive compound is produced and delivered to the organism. Also disclosed herein is an *in vitro* method for producing a issue having *in vivo*-like gross and cellular morphology that involves providing precursor cells of the tissue; mixing the cells with a solution of extracellular matrix components to create a suspension; placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross and cellular morphology of the tissue and having attachment surfaces coupled thereto, allowing the suspension to coalesce; and culturing the cells under conditions in which the cells form an organized tissue connected to the attachment surfaces. Also disclosed herein is an apparatus for producing *in vitro* a tissue having *in vivo*-like gross and cellular morphology. This apparatus includes a vessel having a three-dimensional geometry approximating the *in vivo* morphology of the tissue and tissue attachment surfaces coupled thereto.

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DELIVERY OF BIOACTIVE COMPOUNDS TO AN ORGANISM

Inventor: Herman H. Vandenburgh

Background of the Invention

This invention relates to the delivery of bioactive compounds to an organism, and in particular to methods and apparatus for the delivery of bioactive compounds by implanting into the organism an organized tissue producing the compounds.

One of the primary therapies used to treat disease is the delivery of bioactive compounds to the affected organism. Bioactive compounds may be delivered systemically or locally by a wide of variety of methods. For example, an exogenous source (i.e., produced outside the organism treated) of the bioactive compound may be provided intermittently by repeated doses. The route of administration may include oral consumption, injection, or tissue absorption via topical compositions, suppositories, inhalants, or the like. Exogenous sources of the bioactive compound may also be provided continuously over a defined time period. For example, delivery systems such as pumps, time-released compositions, or the like may be implanted into the organism on a semi-permanent basis for the administration of bioactive compounds (e.g., insulin, estrogen, progesterone, etc.).

The delivery of bioactive compounds from an endogenous source (i.e., produced within the organism treated) has also been attempted. Traditionally, this was accomplished by transplanting, from another organism, an organ or tissue whose normal physiological function was the production of the bioactive compound (e.g., liver transplantation, kidney transplantation, or the like). More recently, endogenous production by cells of the affected organism has been accomplished by inserting into the cells a DNA sequence which mediates the production of

the bioactive compound. Commonly known as gene therapy, this method includes inserting the DNA sequence into the cells of the organism *in vivo*. The DNA sequence persists either transiently or permanently as an extra-chromosomal vector (e.g., when inserted by adenovirus infection or by direct injection of a plasmid) or integrates into the host cell genome (e.g., when inserted by retrovirus infection). Alternatively, the DNA sequence may be inserted into cells of the host tissue or an another organism *in vitro*, and the cells subsequently transplanted into the organism to be treated.

Summary of the Invention

In general, the invention features a method of delivering a bioactive compound to an organism. The method includes the steps of growing a plurality of cells *in vitro* under conditions that allow the formation of an organized tissue, at least a subset of the cells containing a foreign DNA sequence which mediates the production of the bioactive compound, and implanting the cells into the organism, whereby the bioactive compound is produced and delivered to the organism.

In a preferred embodiment of this method, the step of growing may include mixing the cells with a solution of extracellular matrix components to create a suspension, placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross morphology of the tissue and having tissue attachments surfaces thereon, allowing the suspension to coalesce, and culturing the coalesced suspension under conditions in which the cells connect to the attachment surfaces and form a tissue having an *in vivo*-like gross and cellular morphology.

In other preferred embodiments, the DNA sequence encodes the bioactive compound; the DNA sequence encodes

a protein which mediates the production of the bioactive compound (for example, by regulating its expression or encoding an intermediate to the bioactive compound); the DNA sequence mediates the production of two bioactive compounds; the tissue includes skeletal muscle; the tissue includes myotubes; the bioactive compound is a growth factor (for example, human growth hormone); the bioactive compound is a bone morphogenetic protein; the bone morphogenetic protein is BMP-6; the organized tissue is implanted into the tissue of origin of at least one of the cells; the cells include a first and a second population of cells, at least a subset of each of the populations containing a foreign DNA sequence which mediates the production of a bioactive compound; the foreign DNA sequence of the first population mediates the production of a bioactive compound different from the foreign DNA sequence of the second population; and the foreign DNA sequence of the first population encodes a bone morphogenetic protein and the foreign DNA sequence of the second population includes a parathyroid hormone.

In other preferred embodiments, the method includes: the step of removing the organized tissue from the organism to terminate delivery of the bioactive compound; following the removal step, the step of culturing the organized tissue *in vitro* under conditions which preserve its *in vivo* viability; following the culturing step, the step of reimplanting the organized tissue into the organism to deliver the bioactive compound to the organism; the step of isolating primary cell types of at least one of the cell types of the tissue; and the step of utilizing immortalized cells of at least one of the cell types of the tissue.

In other preferred embodiments of this method, the tissue comprises substantially post-mitotic cells; during the growing step, a force is exerted substantially

parallel to a dimension of the tissue; the force is exerted on the individual cells during growth *in vitro* and on the organized tissue during implantation *in vivo*; the coalesced suspension exerts a force on the cells substantially parallel to a dimension of the vessel; the cells are aligned substantially parallel to a dimension of the vessel; the vessel is substantially semi-cylindrical in shape; the attachment surfaces are positioned at opposite ends of the vessel; the alignment is mediated by forces exerted by the coalesced suspension; the cells comprise myotubes; the organism is a mammal; and the mammal is a human.

In a related aspect, the invention features an organized tissue producing a bioactive compound, the tissue is produced by the steps of mixing a plurality of cells with a solution of extracellular matrix components to create a suspension, at least a subset of the cells containing a foreign DNA sequence which mediates the production of a bioactive compound; placing the suspension in a vessel having a three dimensional geometry approximating the *in vivo* gross morphology of the tissue, the vessel having attachment surfaces thereon; allowing the suspension to coalesce; and culturing the coalesced suspension under conditions in which the cells connect to the attachment surfaces and form a tissue having an *in vivo*-like gross and cellular morphology.

In a related aspect, the invention features an organized tissue producing a bioactive compound. The organized tissue includes a plurality of cells, grown *in vitro* under conditions that allow the formation of an organized tissue, and a foreign DNA sequence mediating the production of a bioactive compound. The DNA sequence is inserted into at least a subset of the cells. Also included in the invention are organized tissues producing

a bioactive compound, the tissue being produced by any of the methods described herein.

In preferred embodiments, the organized tissue is skeletal muscle.

In a related aspect, the invention features an *in vitro* method for producing a tissue having an *in vivo*-like gross and cellular morphology. The method includes providing precursor cells of the tissue; mixing the cells with a solution of extracellular matrix components to create a suspension; placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross morphology of the tissue, the vessel having tissue attachment surfaces thereon; allowing the suspension to coalesce; and culturing the cells under conditions in which the cells form an organized tissue connected to the attachment surfaces.

In preferred embodiments of this method, the step of providing includes isolating primary cells of at least one of the cell types which make up the tissue or includes utilizing immortalized cells of at least one of the cell types which make up the tissue; the step of providing includes inserting a foreign DNA sequence into at least one of the cells which make up the tissue; the tissue includes substantially post-mitotic cells; the coalesced suspension exerts a force on the cells substantially parallel to a dimension of the vessel; the cells are aligned substantially parallel to a dimension of the vessel; the vessel is substantially semi-cylindrical in shape; and the attachment surfaces are positioned at opposite ends of the vessel.

In other preferred embodiments of this method, the DNA sequence encodes the bioactive compound; the DNA sequence encodes a protein which mediates the production of the bioactive compound; the DNA sequence mediates the production of two bioactive compounds; the bioactive

compound is a growth factor; the organized tissue is implanted into the organism, whereby the bioactive compound is produced and delivered to the organism; and the organized tissue is implanted into the tissue of origin of at least one of the cells.

In a related aspect, the invention features an organized tissue produced by the steps of providing precursor cells of the tissue; mixing the cells with a solution of extracellular matrix components to create a suspension; placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross morphology of the tissue, the vessel having tissue attachment surfaces thereon; allowing the suspension to coalesce; and culturing the cells under conditions in which the cells form an organized tissue connected to the attachment surfaces. Also included in the invention are organized tissues produced by any of the methods described herein.

In a related aspect, the invention features an apparatus for producing a tissue *in vitro* having an *in vivo*-like gross and cellular morphology. The apparatus includes a vessel having a three-dimensional geometry approximating the *in vivo* gross morphology of the tissue and having tissue attachment surfaces in the vessel.

In preferred embodiments of this aspect of the invention, the apparatus further includes a culture chamber in which the vessel may be submerged; the vessel is substantially semi-cylindrical in shape; the attachment surfaces are coupled to opposite ends of the semi-cylindrical vessel; the coalesced suspension exerts a force on the cells substantially parallel to a dimension of the vessel; and the cells are aligned substantially parallel to a dimension of the vessel.

In a related aspect, the invention features a method of regulating bone formation in an organism. The

method includes the steps of growing a plurality of cells *in vitro* under conditions that allow the formation of an organized tissue, at least a subset of the cells containing a foreign DNA sequence which mediates the production of a bone morphogenetic protein, and implanting the tissue into the organism, whereby the bone morphogenetic protein is produced and delivered to chondroblastic or osteoblastic precursor cells.

In a preferred embodiment of this method, the step of growing may include mixing the cells with a solution of extracellular matrix components to create a suspension; placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross morphology of the tissue and having tissue attachments surfaces thereon; allowing the suspension to coalesce; and culturing the coalesced suspension under conditions in which the cells connect to the attachment surfaces and form a tissue having an *in vivo*-like gross and cellular morphology.

In other preferred embodiments, the DNA sequence encodes the bone morphogenetic protein; the DNA sequence encodes BMP-6; the DNA sequence encodes a protein which mediates the production of the bone morphogenetic protein (for example, by regulating its expression or encoding an intermediate to the bioactive compound); the DNA sequence also mediates the production of another bioactive compound; the tissue includes skeletal muscle; the tissue includes myotubes; the bioactive compound is a growth factor (for example, human growth hormone); the organized tissue is implanted into the tissue of origin of at least one of the cells; the cells include a first and a second population of cells, at least a subset of each of the populations containing a foreign DNA sequence which mediates the production of a bioactive compound; the foreign DNA sequence of the first population mediates the

production of a bioactive compound different from the foreign DNA sequence of the second population; and the foreign DNA sequence of the first population encodes a bone morphogenetic protein and the foreign DNA sequence of the second population includes a parathyroid hormone.

In other preferred embodiments, the method includes: the step of removing the organized tissue from the organism to terminate delivery of the bone morphogenetic protein; following the removal step, the step of culturing the organized tissue *in vitro* under conditions which preserve its *in vivo* viability; following the culturing step, the step of reimplanting the organized tissue into the organism to deliver the bone morphogenetic protein to the organism; the step of isolating primary cell types of at least one of the cell types of the tissue; and the step of utilizing immortalized cells of at least one of the cell types of the tissue.

In other preferred embodiments of this method, the tissue comprises substantially post-mitotic cells; during the growing step, a force is exerted substantially parallel to a dimension of the tissue; the force is exerted on the individual cells during growth *in vitro* and on the organized tissue during implantation *in vivo*; the coalesced suspension exerts a force on the cells substantially parallel to a dimension of the vessel; the cells are aligned substantially parallel to a dimension of the vessel; the vessel is substantially semi-cylindrical in shape; the attachment surfaces are positioned at opposite ends of the vessel; the alignment is mediated by forces exerted by the coalesced suspension; the cells comprise myotubes; the organism is a mammal; and the mammal is a human.

As used herein, by a "bioactive compound" is meant a compound which influences the biological structure,

function, or activity of a cell or tissue of a living organism.

By "bone morphogenetic protein" is meant an extracellular osteogenic-stimulating molecule belonging to the TGF-β superfamily. Bone morphogenetic proteins ("BMP") include a large number of proteins, for example, BMP-2, -3, -4, -5, -6, -7, -11, and -12. Bone morphogenetic proteins control the cellular events associated with bone and cartilage formation and repair (e.g., cellular growth, proliferation, and differentiation). For example, bone morphogenetic proteins alter the differentiation pathway of mesenchymal cells towards the chondroblastic or osteoblastic lineage.

By "organized tissue" or "organoid" is meant a tissue formed *in vitro* from a collection of cells having a cellular organization and gross morphology similar to that of the tissue of origin for at least a subset of the cells in the collection. An organized tissue or organoid may include a mixture of different cells, for example, muscle, fibroblast, and nerve cells, but must exhibit the *in vivo* cellular organization and gross morphology of a tissue including at least one of those cells, for example, the organization and morphology of muscle tissue.

By "*in vivo*-like gross and cellular morphology" is meant a three-dimensional shape and cellular organization substantially similar to that of the tissue *in vivo*.

By "extracellular matrix components" is meant compounds, whether natural or synthetic compounds, which function as substrates for cell attachment and growth. Examples of extracellular matrix components include, without limitation, collagen, laminin, fibronectin, vitronectin, elastin, glycosaminoglycans, proteoglycans, and combinations of some or all of these components

(e.g., Matrigel™, Collaborative Research, Catalog No. 40234).

By "tissue attachment surfaces" is meant surfaces having a texture, charge or coating to which cells may adhere *in vitro*. Examples of attachment surfaces include, without limitation, stainless steel wire, VELCRO™, suturing material, native tendon, covalently modified plastics (e.g., RGD complex), and silicon rubber tubing having a textured surface.

By "foreign DNA sequence" is meant a DNA sequence which differs from that of the wild type genomic DNA of the organism and may be extra-chromosomal, integrated into the chromosome, or the result of a mutation in the genomic DNA sequence.

By "substantially post-mitotic cells" is meant an organoid in which at least 50% of the cells containing a foreign DNA sequence are non-proliferative. Preferably, organoids including substantially post-mitotic cells are those in which at least 80% of the cells containing a foreign DNA sequence are non-proliferative. More preferably, organoids including substantially post-mitotic cells are those in which at least 90% of the cells containing a foreign DNA sequence are non-proliferative. Most preferably, organoids including substantially post-mitotic cells are those in which 99% of the cells containing a foreign DNA sequence are non-proliferative. Cells of an organoid retaining proliferative capacity may include cells of any of the types included in the tissue. For example, in skeletal muscle organoids, the proliferative cells may include muscle stem cells (i.e., satellite cells) and fibroblasts.

The invention provides a number of advantages. For example, implantation of an organized tissue produced *in vitro* provides quantifiable, reproducible, and

localized delivery of bioactive compounds to an organism. Prior to implantation, the production of bioactive compounds by the organized tissue may be measured and quantified per unit time, per unit mass, or relative to any other physiologically-relevant parameter. In addition, the capability of an organized tissue to sustain production of bioactive compounds can be assessed by culturing for extended periods and assaying of compound production with time.

Moreover, because the organized tissue is implanted at a defined anatomical location as a discrete collection of cells, it may be distinguished from host tissues, removed post-implantation from the organism, and reimplanted into the organism at the same or a different location at the time of removal or following an interim period of culturing *in vitro*. This feature facilitates transient or localized delivery of the bioactive compound. Restriction of the cells producing bioactive compounds to particular anatomical sites also enhances the controlled delivery of bioactive compounds, especially where the organized tissue functions as a paracrine organ. The efficiency of delivery of a bioactive compound (i.e., the amount of the bioactive compound delivered to obtain a desired serum concentration) is also enhanced as compared to direct subcutaneous injection. Likewise, the efficiency of implanting post-mitotic cells containing a foreign DNA sequence into an organism (i.e., the number of cells in a post-mitotic state as a percentage of the initial number of cells containing the foreign DNA sequence) is enhanced by organoid implantation as compared to the implantation of individual mitotic cells. For example, skeletal muscle organoids produced *in vitro* include post-mitotic myofibers representing greater than 70% of the initial myoblasts containing a foreign DNA sequence, whereas

direct implantation of the myoblasts results in post-mitotic myofibers representing less than 1% of the initial cells.

In addition, because substantially all of the implanted cells are fully differentiated, migration of these cells to other anatomical sites is reduced. Moreover, implantation of post-mitotic, non-migratory myofibers containing a foreign DNA reduces the possibility of cell transformation and tumor formation. The implantation of an organized tissue may even enhance the functional and structural characteristics of the host tissue.

Furthermore, because the method of producing a tissue having an *in vivo*-like gross and cellular morphology may be achieved without the application of external forces by mechanical devices, the apparatus for producing such a tissue is readily adaptable to standard cell and tissue culture systems. The apparatus and method may also be used to produce bone, cartilage, tendon, and cardiac tissues as these tissues include cell types which organize in response to external forces. In addition, the apparatus includes widely available, easily assembled and relatively inexpensive components.

Other advantages and features of the invention will be apparent from the detailed description, and from the claims.

Brief Description of the Drawings

FIG. 1 is a diagram of a vessel for growing skeletal muscle tissue which will have an *in vivo*-like gross and cellular morphology.

FIG. 2 is a flow chart of the process of skeletal muscle growth and regeneration.

FIG. 3 is a photograph of skeletal muscle organoids formed *in vitro* from rhGH-secreting C2C12 cells.

FIG. 4 is a micrograph of a section of a skeletal muscle organoid grown *in vitro* from rhGH-secreting C2C12 cells which has been stained for sarcomeric tropomyosin.

FIG. 5 is a micrograph of a section of a skeletal muscle organoid grown *in vitro* from rhGH-secreting C2C12 cells which has been stained for sarcomeric tropomyosin.

FIG. 6 is a flow chart comparing myoblast and myofiber gene therapy methods.

FIG. 7A-7F are graphs of rhGH serum levels in mice following skeletal muscle organoid implantation.

FIG. 8A-8B are graphs of the effects of cytosine arabinoside on rhGH-secreting C2C12 proliferating myoblasts and post-mitotic myofibers.

FIG. 9A-9C are photographs of a skeletal muscle organoid grown *in vitro* from rhGH-secreting C2C12 cells, implanted *in vivo*, and subsequently removed and further cultured *in vitro*.

Fig. 10A-10C are Northern blots of rhBMP-6 mRNA levels in C2C12 cells retrovirally-transduced with a rhBMP-6 gene.

Fig. 11 is a graph of alkaline phosphatase activity in controls and C2C12 cells retrovirally-transduced with a rhBMP-6 gene.

Fig. 12A and 12B are micrographs of C2C12 cells retrovirally-transduced with a rhBMP-6 gene which have been stained for sarcomeric tropomyosin.

Detailed Description

I. *In Vitro* Production of Tissues Having *In Vivo*-like Gross and Cellular Morphology

Organized tissues having *in vivo*-like gross and cellular morphology may be produced *in vitro* from the individual cells of a tissue of interest. As a first step in this process, disaggregated or partially

disaggregated cells are mixed with a solution of extracellular matrix components to create a suspension. This suspension is then placed in a vessel having a three dimensional geometry which approximates the *in vivo* gross morphology of the tissue and includes tissue attachment surfaces coupled to the vessel. The cells and extracellular matrix components are then allowed to coalesce or gel within the vessel, and the vessel is placed within a culture chamber and surrounded with media under conditions in which the cells are allowed to form an organized tissue connected to the attachment surfaces.

Although this method is compatible with the *in vitro* production of a wide variety of tissues, it is particularly suitable for tissues in which at least a subset of the individual cells are exposed to and impacted by mechanical forces during tissue development, remodeling or normal physiologic function. Examples of such tissues include muscle, bone, skin, nerve, tendon, cartilage, connective tissue, endothelial tissue, epithelial tissue, and lung. More specific examples include skeletal, cardiac, and smooth muscle, stratified or lamellar bone, and hyaline cartilage. Where the tissue includes a plurality of cell types, the different types of cells may be obtained from the same or different organisms, the same or different donors, and the same or different tissues. Moreover, the cells may be primary cells or immortalized cells. Furthermore, all or some of the cells of the tissue may contain a foreign DNA sequence which mediates the production of a bioactive compound (as described herein).

The composition of the solution of extracellular matrix components will vary according to the tissue produced. Representative extracellular matrix components include, but are not limited to, collagen, laminin, fibronectin, vitronectin, elastin, glycosaminoglycans,

proteoglycans, and combinations of some or all of these components (e.g., Matrigel™, Collaborative Research, Catalog No. 40234). In tissues containing cell types which are responsive to mechanical forces, the solution of extracellular matrix components preferably gels or coalesces such that the cells are exposed to forces associated with the internal tension in the gel.

Culture conditions will also vary according to the tissue produced. Methods for culturing cells are well known in the art and are described, for example, in Animal Cell Culture: A Practical Approach, (R.I. Fveshney, ed. IRL Press, 1986). In general, the vessel containing a coalesced suspension of cells and extracellular matrix components is placed in a standard culture chamber (e.g., wells, dishes, or the like), and the chamber is then filled with culture medium until the vessel is submerged. The composition of the culture medium is varied, for example, according to the tissue produced, the necessity of controlling the proliferation or differentiation of some or all of the cells in the tissue, the length of the culture period and the requirement for particular constituents to mediate the production of a particular bioactive compound. The culture vessel may be constructed from a variety of materials in a variety of shapes as described below.

An apparatus for producing a tissue *in vitro* having an *in vivo*-like gross and cellular morphology includes a vessel having a three dimensional geometry which approximates the *in vivo* gross morphology of the tissue. The apparatus also includes tissue attachment surfaces coupled to the vessel. Such a vessel may be constructed from a variety of materials which are compatible with the culturing of cells and tissues (e.g., capable of being sterilized and compatible with a particular solution of extracellular matrix components)

and which are formable into three dimensional shapes approximating the *in vivo* gross morphology of a tissue of interest. The tissue attachment surfaces (e.g., stainless steel mesh, VELCRO™, or the like) are coupled to the vessel and positioned such that as the tissue forms *in vitro* the cells may adhere to and align between the attachment surfaces. The tissue attachment surfaces may be constructed from a variety of materials which are compatible with the culturing of cells and tissues (e.g., capable of being sterilized, or having an appropriate surface charge, texture, or coating for cell adherence).

The tissue attachment surfaces may be coupled in a variety of ways to an interior or exterior surface of the vessel. Alternatively, the tissue attachment surfaces may be coupled to the culture chamber such that they are positioned adjacent the vessel and accessible by the cells during tissue formation. In addition to serving as points of adherence, in certain tissue types (e.g., muscle), the attachment surfaces allow for the development of tension by the tissue between opposing attachment surfaces. Moreover, where it is desirable to maintain this tension *in vivo*, the tissue attachment surfaces may be implanted into an organism along with the tissue (see further discussion in Section II.).

One vessel according to the invention is shown in Fig. 1. This vessel 1, which is suitable for the *in vitro* production of a skeletal muscle organoid 3, has a substantially semi-cylindrical shape and tissue attachment surfaces 2 coupled to an interior surface of the vessel.

A. *In Vitro* Production of a Skeletal Muscle Organoid <u>having *In Vivo*-Like Gross and Cellular Morphology</u>

Using an apparatus and method as generally described above, a skeletal muscle organoid having an *in*

vivo-like gross and cellular morphology was produced *in vitro*. An overview of the stages of skeletal muscle growth and regeneration is shown in Fig. 2. As shown, during skeletal muscle development embryonic myoblasts proliferate, differentiate, and then fuse to form multi-nucleated myofibers. Although the myofibers are non-proliferative, a population of muscle stem cells (i.e., satellite cells), derived from the embryonic myoblast precursor cells, retain their proliferative capacity and serve as a source of myoblasts for muscle regeneration in the adult organism. Therefore, either embryonic myoblasts or adult skeletal muscle stem cells may serve as one of the types of precursor cells for *in vitro* production of a skeletal muscle organoid.

To produce skeletal muscle cells capable of secreting a bioactive compound, primary rat or avian cells or immortalized murine cells secreting recombinant human growth hormone, were suspended in a solution of collagen and Matrigel™ which was maintained at 4°C to prevent gelling. The cell suspension was then placed in a semi-cylindrical vessel with tissue attachment surfaces coupled to an interior surface at each end of the vessel. The vessel was positioned in the bottom of a standard cell culture chamber. Following two to four hours of incubation at 37°C, the gelled cell suspension was covered with fresh culture medium (renewed at 24 to 72 hour intervals) and the chamber containing the suspended cells was maintained in a humidified 5% CO_2 incubator at 37°C throughout the experiment.

Between the second and sixth day of culture, the cells were found to be organized to the extent that they spontaneously detached from the vessel. At this stage, the cells were suspended in culture medium while coupled under tension between tissue attachment surfaces positioned at either end of the culture vessel. During

the subsequent ten to fourteen days, the cells formed an organoid containing skeletal myofibers aligned parallel to each other in three dimensions. The alignment of the myofibers and the gross and cellular morphology of the organoid were similar to that of *in vivo* skeletal muscle.

To carry out the above method, an apparatus for organoid formation was constructed from silastic tubing and either VELCRO™ or metal screens as follows. A section of silastic tubing (approximately 5 mm I.D., 8 mm O.D., and 30 mm length) was split in half with a razor blade and sealed at each end with silicone rubber caulking. Strips of VELCRO™ (loop or hook side, 3 mm wide by 4 mm long) or L-shaped strips of stainless steel screen (3 mm wide by 4 mm long by 4 mm high) were then attached with silicone rubber caulking to the interior surface of the split tubing near the sealed ends. The apparatus was thoroughly rinsed with distilled/deionized water and subjected to gas sterilization.

Skeletal muscle organoids were produced *in vitro* from a C2C12 mouse skeletal muscle myoblast cell line stably co-transfected with recombinant human growth hormone-expressing and β-galactosidase-expressing (β-gal) constructs. Dhawan et al., *Science* 254:1509-1512, 1991. Cells were plated in the vessel at a density of 1-4 X 10^6 cells per vessel in 400 μl of a solution containing extracellular matrix components. The suspension of cells and extracellular matrix components was achieved by the following method. The solution includes 1 part Matrigel™ (Collaborative Research, Catalog No. 40234) and 6 parts of a 1.6 mg/ml solution of rat tail Type I collagen (Collaborative Research, Catalog No. 40236). The Matrigel™ was defrosted slowly on ice and kept chilled until use. The collagen solution was prepared just prior to cell plating by adding to lyophilized collagen, growth medium (see constituents below), and 0.1N NaOH in volumes

equivalent to 90% and 10%, respectively, of the volume required to obtain a final concentration of 1.6 mg/ml and a pH of 7.0-7.3. The collagen, sodium hydroxide and growth medium were maintained on ice prior to and after mixing by inversion.

Freshly centrifuged cells were suspended in the collagen solution by trituration with a chilled sterile pipet. Matrigel™ was subsequently added with a chilled pipet and the suspension was once again mixed by trituration. The suspension of cells and extracellular matrix components was maintained on ice until it was plated in the vessel using chilled pipet tips. The solution was pipetted and spread along the length of the vessel, taking care to integrate the solution into the tissue attachment surfaces. The culture chamber containing the vessel was then placed in a standard cell culture incubator, taking care not to shake or disturb the suspension. The suspension was allowed to gel, and after 2 hours the culture chamber was filled with growth medium such that the vessel was submerged.

For a period of three days the cells were maintained on growth medium containing DMEM-high glucose (GIBCO-BRL), 5% fetal calf serum (Hyclone Laboratories), and 1% penicillin/streptomycin solution (final concentration 100 units/ml and 0.1 μg/ml, respectively). On the fourth day of culture, the cells were switched to fusion medium containing DMEM-high glucose, 2% horse serum (Hyclone Laboratories), and 100 units/ml penicillin for a period of 4 days. On the eighth day of culture, the cells were switched to maintenance medium containing DMEM-high glucose, 10% horse serum, 5% fetal calf serum, and 100 units/ml penicillin for the remainder of the experiment. Before the organoids were ready for implantation, some were cultured in maintenance media containing 1 μg/ml of cytosine arabinoside for the final

four to eight days. Treatment with cytosine arabinoside eliminated proliferating cells and produced organoids including substantially post-mitotic cells.

The cell-extracellular matrix gel (cell-gel) formed *in vitro* from these stably transfected C2C12 cells 48 hours after plating are shown in Fig. 3. In the upper half of the figure the cell-gel has detached from one of the tissue attachment surfaces. The resultant contraction demonstrates the tension developed in the gel between the tissue attachment surfaces. Figs. 4 and 5 demonstrate the presence of a muscle-specific contractile protein (i.e., brown staining following incubation with an antibody to sarcomeric tropomyosin), in parallel arrays of highly organized and longitudinally oriented myofibers in mammalian skeletal muscle organoids following three weeks of culturing in the apparatus shown in Fig. 1. Moreover, Fig. 9B shows the retention of myofiber organization following organoid implantation.

II. Delivery of Bioactive Compounds

Bioactive compounds may be delivered to an organism by growing individual cells *in vitro* under conditions that result in the formation of an organized tissue producing the bioactive compound and subsequently implanting the organized tissue into the organism (see Section I. for detailed description of organized tissue production). Production of the bioactive compound by the organized tissue is mediated by a foreign DNA sequence present in at least a subset of the cells which make up the implanted tissue.

A variety of bioactive compounds may be delivered by this method, and they may function through intracellular (i.e., within the cells of the organized tissue or organoid), endocrine, autocrine, or paracrine mechanisms. Moreover, the organoid may deliver multiple

bioactive compounds either simultaneously or sequentially (e.g., one bioactive compound mediates the delivery of another). Liberation of the bioactive compound from the cells of the organoid may occur by either passive or active processes (e.g., diffusion or secretion).

For example, the bioactive compound may be a hormone, growth factor, or the like which is produced and liberated by the cells of the organoid to act locally or systemically on host tissues. Alternatively, the bioactive compound may function within the cells or on the surface of the cells of the organoid to enhance the uptake or metabolism of compounds from the host tissue or circulation (e.g., lactic acid, low density lipoprotein). Where the organoid serves as a functional and structural adjunct to the host tissue, delivery of growth factors by autocrine or paracrine mechanisms may enhance the integration of the organoid into host tissues. Similarly, where multiple bioactive compounds are produced by the organoid, autocrine delivery of one of the bioactive compounds may be used to regulate the production of one or more of the other bioactive compounds.

The organoid may be implanted by standard laboratory or surgical techniques at a desired anatomical location within the organism. For example, the organoid may be implanted in the same or a different tissue from the tissue of origin of at least one of the individual cells. The location of implantation depends, in part, upon the method of delivery and the identity of the particular bioactive compound to be delivered. For example, an organoid acting as an endocrine organ may be implanted in or adjacent a highly vascularized host tissue. Alternatively, an organoid acting as a paracrine organ is preferably implanted in or adjacent to the host

tissue to which the bioactive compound is to be delivered.

The organoid may be implanted by attachment to a host tissue or as a free floating tissue. In addition, attached organoids may be implanted with or without the tissue attachment surfaces used for *in vitro* tissue formation. Tissues responsive to mechanical forces are preferably implanted by attaching directly to the host tissue or by implanting the organoid coupled to the attachment surfaces so that the organoid is exposed to mechanical forces *in vivo*. For example, skeletal muscle organoids are preferably implanted by attachment to the host tissue under tension along a longitudinal axis of the organoid. Moreover, the organoids may be permanently or temporarily implanted. Permanent implantation may be preferred, for example, where the organoid produces a bioactive compound which corrects a systemic metabolic error (e.g., delivery of insulin to treat diabetes), whereas temporary implantation may be preferred where only transient delivery of a bioactive compound is desired (e.g., delivery of a growth factor to enhance wound healing). Furthermore, because organoids may be implanted, removed, and maintained *in vitro* (see Fig. 9A and discussion below), bioactive compounds may be delivered intermittently to the same or a different location in the organism. For example, a skeletal muscle organoid produced from the cells of a human patient (e.g., an autograft) may be implanted at a first anatomical location for a defined period and subsequently implanted at a second location at or after the time of removal.

At least some of the cells of the organoid contain a foreign DNA sequence. The foreign DNA sequence may be extra-chromosomal, integrated into the genomic DNA of the organoid cell, or may result from a mutation in the

genomic DNA of the organoid cell. In addition, the cells of the organoid may contain multiple foreign DNA sequences. Moreover, the different cells of the organoid may contain different foreign DNA sequences. For example, in one embodiment, a skeletal muscle organoid may include myofibers containing a first foreign DNA sequence and fibroblasts containing a second foreign DNA sequence. Alternatively, the skeletal muscle organoid could include myoblasts from different cell lines, each cell line expressing a foreign DNA sequence encoding a different bioactive compound. These "mosaic" organoids allow the combined and/or synergistic effects of particular bioactive compounds to be exploited. For example, myoblasts expressing growth hormone may be combined with myoblasts expressing an insulin-like growth factor to produce organoids useful in stimulating muscle growth/regeneration. Similarly, myoblasts expressing a bone morphogenetic protein may be combined with myoblasts expressing a parathyroid hormone to produce organoids useful in stimulating bone and cartilage growth/regeneration.

In a preferred embodiment, the foreign DNA sequence encodes a protein which is the bioactive compound. The protein is produced by the cells and liberated from the organoid. Alternatively, the DNA sequence may encode an enzyme which mediates the production of a bioactive compound or a cell surface protein which enhances the uptake and metabolism of compounds from the host tissue or circulation (e.g., lactic acid or low density lipoproteins). The DNA sequence may also encode a DNA binding protein which regulates the transcription of the sequence encoding a bioactive compound or an anti-sense RNA which mediates translation of the mRNA for the bioactive compound. The DNA sequence may also bind trans-acting factors such that

the transcription of the sequence (i.e., foreign or native) encoding the bioactive compound is enhanced (e.g., by disinhibition). Furthermore, the foreign DNA sequence may be a cis-acting control element such as a promoter or an enhancer coupled to a native or foreign coding sequence for the bioactive compound or for an enzyme which mediates the production of the bioactive compound.

A. Delivery of Human Growth Hormone to Mice by Implanting Skeletal Muscle Organoids

Figure 6 shows an overview and comparison of myoblast and myofiber gene therapy. Both methods generally involve isolating myoblasts from a patient in need of gene therapy, inserting into the myoblasts a DNA sequence encoding a bioactive compound, and expanding the myoblast cell population by *in vitro* culturing. In contrast to myoblast gene therapy, the myoblasts used in myofiber gene therapy are further cultured *in vitro* under conditions which result in the formation of an organoid having *in vivo*-like gross and cellular morphology. The organoid is subsequently implanted into the patient to deliver the bioactive compound.

To carry out the delivery of a bioactive compound to an organism, skeletal muscle organoids were formed *in vitro*, as described above, from C2C12 mouse skeletal muscle myoblasts stably co-transfected with recombinant human growth hormone-expressing and β-galactosidase-expressing constructs. Prior to implantation, *in vitro* production of recombinant human growth hormone ("rhGH") was measured by radioimmunoassay according to the manufacturer's instructions (Nichols Institute Diagnostics, San Juan Capistrano, CA). Between three and twenty-four days of culture, the mean rhGH production

ranged between 1.0 and 3.5 μg/day/organoid (see Table 1).

TABLE 1

IN VITRO PREIMPLANT SUMMARY

Experiment	Date	Initial Cell # per Organoid (x 10^4)	Age of Organoid (Days)	Mean rhGH (μg/day/ organoid) (N=)	Treatment of Organoids
IMPLANT 1	8/24	6	3 7	1.9 (2) 3.5 (2)	none
IMPLANT 2	9/21	-	-	-	-
IMPLANT 3	10/5	4	7 12	1.7 - 2.8 (7) 1.9 - 2.5 (6)	none
IMPLANT 4	10/20	2	21	2.7 - 3.6 (5)	none
IMPLANT 5	10/25	2	12 12	2.9 (12) 2.0 (4)	no cytosine arabinoside ("araC") 1 ug/ml araC for 4 days
IMPLANT 6	11/8	3	19	1.9 (6) 1.0 (6)	no araC 1 ug/ml araC for 5 days
IMPLANT 7	11/9	3 (non-rhGH secreting)	1	0 (3)	control experiment
IMPLANT 8	11/3	2	14-20	1.5 to 2.2 (6) 1.2 to 1.6 (6)	no araC 1 ug/ml araC for 5 days
IMPLANT 9	11/30	1 - 2	34	1.7 to 2.4(8)	1 ug/ml araC for 8 days
IMPLANT 10	12/5	1.5 - 2.0	28	2.1 to 2.9 (14)	1 ug/ml araC for 4 days

The organoids were implanted into adult C3HeB/FeJ mice (i.e., syngeneic to C2C12 cells) by the following method. Mice were weighed to determine dosages of cyclosporine and anesthetic. One hour prior to the surgical implantation of the organoid, each mouse was given an injection of 60 mg/kg of cyclosporine A. Each mouse was then selected in turn and anesthetized by intramuscular injection of 55 mg/kg Ketamine, 1 mg/kg Promazine, and 5 mg/kg Xylazine. The site of implantation was then depilated with Nair™ or by

shaving, and prepped for aseptic surgery. For organoids implanted subcutaneously, a four to six centimeter long incision was made along the back, the organoid was implanted in either a free floating state or fixed under tension (e.g., attached to the tissue attachment surfaces), and the incision was closed with four to six sutures of 4.0-black silk.

For organoids implanted intramuscularly, a 15 to 30 millimeter incision was made parallel to the anterior tibialis muscle (e.g., anteriolateral aspect of the lower hind limb) to provide access to the muscle sheath. The anterior tibialis was gently split with forceps from tendon to tendon parallel to the muscle belly, thus providing a cavity for insertion of the organoid. The organoid was carefully removed from the vessel by prying the ends off the tissue attachment surfaces with sterile forceps and inserting it, under resting tension, in the implantation site. The incision was closed as described above. Mice were then followed post-surgically for distress and upon regaining consciousness were returned to an animal care facility. Cyclosporine injections are repeated daily for the duration of the experiment. The experimental protocol for the implantation of skeletal muscle organoids is summarized in Table 2 below.

TABLE 2

IN VIVO PROTOCOL SUMMARY

Experiment	Date	Site of Implant	# of Surviving Animals	rhGH Producers (# and method of implant)
IMPLANT 1	8/24	intramuscular free-floating	2 of 2	0 (1 free)
IMPLANT 2	9/21	controls only - cyclosporine dose-response	6 of 6	no organoids implanted
IMPLANT 3	10/5	subcutaneous free-floating	3 of 4	1 (3C - 2 free)
IMPLANT 4	10/20	subcutaneous fixed under tension	2 of 3	2 (2D - 2 fixed) (3D- 1 fixed/1 free)
IMPLANT 5	10/25	subcutaneous fixed under tension	1 of 2	1 (1E - 3 fixed)
IMPLANT 6	11/8	subcutaneous fixed under tension	4 of 7	3 (6A, 6D, 6E - 1 fixed) (6G - no organoid)
IMPLANT 7	11/9	subcutaneous fixed under tension	2 of 3	0 (7A and 7C - 1 fixed, non-rhGH secreting organoid)
IMPLANT 8	11/13	subcutaneous fixed under tension	5 of 8	4 (8C, 8D, 8F and 8G - 1 fixed)
IMPLANT 9	11/30	subcutaneous fixed under tension or free-floating	7 of 7	5 (9A, 9B, 9C, 9D and 9F - 1 fixed) 1 (9E - 1 free) (9G - no organoid)
IMPLANT 10	12/2	subcutaneous fixed under tension	7 of 11	7 (10A, 10B, 10C, 10D, 10F, 10G, and 10I - 1 fixed)

Blood was collected every one to seven days by tail bleeding from the mice. Sera concentrations of rhGH were measured by radioimmunoassay according to the manufacturer's instructions (Nichols Institute Diagnostics, San Juan Capistrano, CA).

As shown in Figs. 7A-7F, rhGH was detected in the blood of animals receiving rhGH organoid implants, but not in controls (6G, 7A, 7C, and 9G) for up to thirty-three days post-implantation. Serum concentrations were

elevated as high as approximately 5.5 to 9 ng/ml in animals receiving multiple implants of rhGH producing organoids (1E, 2D), whereas serum from animals receiving no implant (6G, 9G) or implants of non-rhGH secreting organoids (7A and 7C) contained no detectable rhGH. In addition, animals receiving organoids treated *in vitro* with cytosine arabinoside prior to implantation (1E, 6E, 8D, 8F, 8G, 9A through 9F, and 10A through 10J) demonstrated serum rhGH levels comparable to those of animals receiving implants which were not treated *in vitro* with cytosine arabinoside prior to implantation (i.e., 2D, 3C, 3D, 6A, 6D, and 8C). Under the conditions used in this study, cytosine arabinoside treatment kills greater than 99% of proliferating C2C12 myoblasts while having only a minor effect on myofiber metabolism and rhGH secretion (Fig. 8). Moreover, Fig. 9C shows that the rhGH gene and the β-galactosidase gene are only expressed in post-mitotic myofibers. These results demonstrate that organoids including substantially post-mitotic cells can deliver therapeutic levels of a bioactive compound for up to thirty-three days post-implantation.

It is noteworthy that within forty-eight hours following the removal of implants (i.e., 8D, 8G and 9F), rhGH was undetectable in the sera of animals previously having serum concentrations as high as 2.6 ng/ml. These data demonstrate the reversibility of delivering bioactive compounds by this method. In addition, organoids removed from animals may be re-incubated *in vitro* (see e.g., Fig. 9A). For example, the two organoids implanted into animal 3D produced 188 ng/day of rhGH *in vitro* post-implantation. These data suggest the feasibility of removing organoids and subsequently reimplanting them such that bioactive compounds may be delivered during multiple treatment periods separated in

time. Moreover, the data suggest the feasibility of transplanting sequentially, at different sites within the same organism, organoids functioning as paracrine organs.

The rhGH production of 188 ng/day *in vitro* by organoids from animal 3D and the *in vivo* serum levels of 1.0 ng/ml on day twenty-four (i.e., just prior to removal) suggest a 188-fold difference between organoid production and steady state circulating levels of rhGH in the animal. These results compare favorably to the 500-fold difference between rhGH concentrations delivered by direct subcutaneous injection and steady state circulating levels, (Yang et al. *Circulation* 92:262-267, 1995 (1000 μg/day rhGH by direct subcutaneous injection produced 2 μg/ml serum concentrations in rats). It is also noteworthy that the organoid maintained *in vivo* under tension produced approximately 144 ng/ml when placed *in vitro* on removal from the animal, while the free floating organoid produced only 40 ng/ml when placed *in vitro* on removal from the animal. In addition, an organoid implanted under no tension (9E) was a poorer producer of rhGH *in vivo* than those placed under tension (9A, 9B, 9C). These results suggest that maintaining organoids under tension enhances the production and delivery of bioactive compounds.

B. Delivery of Bone Morphogenetic Protein to an Organism by Implanting Skeletal Muscle Organoids

1. Transduction and Selection of C2C12 Myoblasts Expressing rhBMP-6

φ2 packaging cells producing high titers (> 1 x 10^7 pfu) of retrovirus containing the pLX(rhBMP-6)SN expression vector were provided by Dr. Vladimir Drozdoff, Department of Medicine, Vanderbilt University. Myoblast

cell cultures, 50% confluent in T-75 flasks, were incubated for eight hours in 20 ml of conditioned media from the high viral titer packaging cells. The media was supplemented with 4 μg/ml of polybreen. After eight hours, the cells were placed in DMEM + 10% fetal calf serum containing 2 μg/ml of polybreen, and cultured for an additional 48-72 hr, or until the cells had undergone one or two additional divisions. The transduced cells were then harvested, counted, and plated out as single cell clones in four 12-well plates. The single cell clones were selected by culturing in DMEM + 10% fetal calf serum containing 400 μg/ml of G418. Single cell colonies began to appear after 2-3 weeks in culture. These colonies were first expanded to a single T-25 flask, and then expanded to two T-150 flasks which were grown to 90% confluency. The first flask was harvested for storage of cells in liquid nitrogen, and the second flask was processed for total RNA.

Alternatively, myoblasts are transducible by direct incubation with plasmids containing bone morphogenetic protein genes (e.g., mouse BMP-4, Fang, J., et al., *Stimulation of new bone formation by direct transfer of osteogenic plasmid genes*, Proc. Natl. Acad. Sci. U.S.A. 93:5753-5758, 1996; human BMP-1, BMP-2A and BMP-3, Wozney, J.M., et al., *Novel regulators of bone formation: molecular clones and activities*, Science 242:1528-1532, 1988; human BMP-4, Ahrens, M., et al., *Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages*, DNA and Cell Biology 32:871-880, 1993). For example, myoblasts may be successfully transduced by standard calcium phosphate coprecipitation or lipofection.

Northern blot analysis was performed on the cell clones with 20 μg of total or standard RNA per lane

(Figs. 10A-10C). The blots were hybridized with a cDNA probe to rhBMP-6 (supplied by Genetics Institute, Cambridge, MA). Referring to Figs. 10A and 10B, clones expressing high levels of rhBMP-6 mRNA (e.g., cell line 4A1 in lane 13 of Fig. 10B) were expanded and recloned from single cell colonies. Referring to Fig. 10C, subclones of cell line 4A1 were rescreened by Northern blot analysis, and clones 1A1 and 2A2 expressed high levels of rhBMP-6 mRNA relative to the other clones. Cell colonies retaining high expression of rhBMP-6 were harvested and banked in liquid nitrogen.

2. Expression of Biologically Active BMP-6

The biological activity of rhBMP-6 in cell colonies retaining high expression of rhBMP-6 (i.e., C_2-BMP6 cells) was determined by measuring alkaline phosphate activity (i.e., an osteoblastic marker) in the cells after 14 days in culture. Normal C2C12 cells (i.e., non-transduced cells) and C2C12 cells transduced with the LXSN vector alone (i.e., C_2-LXSN cells) were used as controls.

Cells were harvested after 14 days as follows. Wells containing the cells were rinsed with phosphate buffered saline (0.1m, pH 7.4; PBS) and then typsinized with five drops per well of 0.05% trypsin/EDTA solution in PBS. The trypsin/EDTA was neutralized with 500 μl serum-containing media per well, and cells were transferred to microcentrifuge tubes and centrifuged at 900 rpm for four minutes to pellet the cells. Cell pellets were resuspended and lysed in 500 μl of TXM buffer (10 mM Tris HCL; 1.0 mM magnesium chloride; 0.02 mM zinc chloride; 0.1% Triton X-100; and 0.02% sodium azide), and stored at -20°C until assayed or assayed immediately for alkaline phosphate activity as follows.

One hundred microliters of cell lysate, blank (buffer minus substrate), or standard (5 mM p-nitrophenol in buffer) was added to a tube containing 400 µl of alkaline phosphate assay substrate and buffer (0.1 mg glycine; 2.0 mM magnesium chloride; 2 mg/ml p-nitrophenyl phosphate) and incubated at 37°C for 30 min. The reaction was stopped by adding 500 µl of 0.25 N NaOH, and the optical density at 410 nm was read on a spectrophotometer. The total cellular protein in each sample was measured with a Bio-Rad™ protein assay essentially according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA) and alkaline phosphatase activities calculated as follows:

$$\text{Total Alkaline Phosphatase Activity for Sample } \left(\frac{\mu g}{hour}\right) = \frac{(2 \times \text{Sample Optical Density} \times \text{Dilution Factor})}{(\text{Average of Standard Optical Densities})}$$

$$\text{Alkaline Phosphatase Activity } \left(\frac{\mu g/hour}{mg \text{ cellular protein}}\right) = \frac{\text{Total Alkaline Phosphatase Activity for Sample}}{\text{Total Cellular Protein for Sample}}$$

3. Delivery of BMP-6 by Implanting Skeletal Muscle Organoids

The ability of C_2-BMP6 cells to differentiate and fuse to form skeletal muscle myofibers was analyzed by morphometric analysis and expression of the muscle-specific protein sarcomeric tropomyosin after six to fourteen days in culture. Normal C2C12 cells and C_2-LXSN cells were used as controls.

Normal C2C12 cells, C_2-LXSN cells, and C_2-BMP6 cells were cultured separately in T-75 flasks. At 80% confluence, all cell types were individually subcultured and plated into four well-plates (i.e., 15-mm diameter wells pretreated with a collagen spray 1 mg/ml of rat-tail collagen, type I in 1% acetic acid). The cells were plated at a density of 100,000 cells per well in 750 μl of growth medium (DMEM-high glucose; 10% calf serum; 10% fetal calf serum; 100 units/ml penicillin; and 0.1 mg/ml streptomycin) and incubated in a humidified, 37°C, 5% CO_2 atmosphere.

The cells were fed 750 μl warm growth medium per well every 48 hours (i.e., day 2 and day 4 post-plating). Five days post-plating when all groups showed ~100% confluence, the cells were switched to a low serum fusion medium to promote fusion (DMEM-high glucose; 2% horse serum; 100 units/ml penicillin; 0.1 mg/ml streptomycin). The cells were fed fusion medium on days six, eight and ten post-plating. On day 12 post-plating, the cells were switched to a maintenance medium (DMEM-high glucose; 10% horse serum; 5% fetal calf serum; 100 units/ml penicillin; and 0.1 mg/ml streptomycin). The experiment was terminated on day 14.

Plates were fixed for morphometric analysis 6, 8, 12 and 14 days post-plating as follows. Cells were quickly rinsed twice with Eagle's balanced salt solution

(EBSS), fixed with Histochoice™ for thirty minutes at room temperature, and incubated twice for ten-minutes in EBSS. The samples were then stored in fresh EBSS at 4°C until used for immunohistochemical analysis.

From storage, samples were warmed to room temperature and rinsed with phosphate buffer saline (PBS; 10 mM, pH 7.4). Samples were then incubated with the primary antibody, anti-sarcomeric tropomyosin (1:100 dilution) in 0.5% Tween 20/PBS for thirty minutes at room temperature, followed by PBS rinsing. Secondary antibody and avidin biotinylated enzyme steps were performed essentially according to the Vectastain® Elite ABC Kit protocol. Samples were then developed with diaminobenzidine tetrahydrochloride (DAB) reagent to produce a brown precipitate, and then lightly counterstained with hematoxylin.

Referring to Figs. 12A (Day 8 post-plating) and 12B (Day 14 post-plating), the ability of C_2-BMP6 cells to differentiate and fuse to form skeletal muscle myofibers is demonstrated by morphometric analysis (i.e., the presence of longitudinally-oriented multinucleated fibers) and by the presence of sarcomeric tropomyosin (i.e., a muscle-specific protein expressed in differentiated skeletal muscle myofibers but not in undifferentiated, proliferative myoblasts). Because the expression of a biologically active bone morphogenetic protein does not impair the ability of skeletal muscle myoblasts to differentiate and fuse to form skeletal muscle myofibers, skeletal muscle organoids which express bone morphogenetic proteins are produced as described above (see Section I), and are used to deliver bone morphogenetic proteins to an organism also as described above (see Section II).

Because bone morphogenetic proteins are extracellular molecules, skeletal muscle organoid

delivery of the protein may be through endocrine, autocrine, or paracrine mechanisms. In a preferred embodiment, the organoid may function as a paracrine organ to deliver a bone morphogenetic protein to chondroblastic or osteoblastic precursor cells. For example, a skeletal muscle organoid expressing a bone morphogenetic protein may be implanted adjacent a non-union fracture to stimulate endochondral bone formation and repair. Alternatively, a skeletal muscle organoid could be implanted in an organism adjacent skeletal tissues which are susceptible to degeneration and fracture consequent to aging (e.g., the hip joint or spinal column of elderly humans). Similarly, bone morphogenetic protein expressing organoids may be employed to treat systemic or regional osteoporosis (e.g., of the spine, femoral neck, and scapular regions of elderly humans). Skeletal muscle organoids expressing bone morphogenetic proteins may also function to accelerate cartilage repair and the healing of segmental defects or bony fusions.

What is claimed is:

1. A method of delivering a bioactive compound to an organism, comprising the steps of:

growing a plurality of cells *in vitro* under conditions that allow the formation of an organized tissue, at least a subset of said cells containing a foreign DNA sequence which mediates the production of said bioactive compound; and

implanting said tissue into said organism, whereby said bioactive compound is produced and delivered to said organism.

2. The method of claim 1, wherein the step of growing comprises:

mixing said cells with a solution of extracellular matrix components to create a suspension;

placing said suspension in a vessel having a three dimensional geometry approximating the *in vivo* gross morphology of said tissue, said vessel having attachment surfaces thereon;

allowing said suspension to coalesce; and

culturing said coalesced suspension under conditions in which said cells connect to said attachment surfaces and form a tissue having an *in vivo*-like gross and cellular morphology.

3. The method of claim 1, further comprising the steps of:

removing said tissue from said organism to terminate delivery of said bioactive compound.

4. The method of claim 3, further comprising, following said removal step, the step of:

culturing said tissue *in vitro* under conditions which preserve its *in vivo* viability.

5. The method of claim 4, further comprising, following said culturing step, the step of:

reimplanting said tissue into said organism to deliver said bioactive compound to said organism.

6. The method of claim 1, wherein said tissue is implanted into the tissue of origin of at least one of said cells.

7. The method of claim 1, wherein said DNA sequence encodes said bioactive compound.

8. The method of claim 1, wherein said DNA sequence encodes a protein which mediates the production of said bioactive compound.

9. The method of claim 1, wherein said DNA sequence mediates the production of two bioactive compounds.

10. The method of claim 1, wherein said bioactive compound is a growth factor.

11. The method of claim 1, wherein said tissue comprises substantially post-mitotic cells.

12. The method of claim 1, wherein, during said growing step, a force is exerted substantially parallel to a dimension of said tissue.

13. The method of claim 12, wherein said force is exerted on said individual cells during growth *in vitro* and on said organized tissue during implantation *in vivo*.

14. The method of claim 1, wherein said tissue comprises skeletal muscle.

15. The method of claim 1, wherein said tissue comprises myotubes.

16. The method of claim 1, wherein said organism is a mammal.

17. The method of claim 16, wherein said mammal is a human.

18. An organized tissue producing a bioactive compound, said tissue produced by the steps of:

mixing a plurality of cells with a solution of extracellular matrix components to create a suspension, at least a subset of said cells containing a foreign DNA sequence which mediates the production of a bioactive compound;

placing said suspension in a vessel having a three dimensional geometry approximating the *in vivo* gross morphology of said tissue, said vessel having attachment surfaces thereon;

allowing said suspension to coalesce; and

culturing said coalesced suspension under conditions in which said cells connect to said attachment surfaces and form a tissue having an *in vivo*-like gross and cellular morphology.

19. An organized tissue producing a bioactive compound, comprising:

a pluraity of cells grown *in vitro* under conditions that allow the formation of an organized tissue; and

a foreign DNA sequence mediating the production of a bioactive compound, said DNA sequence being inserted into at least a subset of said cells.

20. The organized tissue of claim 19, wherein said tissue is skeletal muscle.

21. An *in vitro* method for producing a tissue having an *in vivo*-like gross and cellular morphology, comprising the steps of:

providing precursor cells of said tissue;

mixing said cells with a solution of extracellular matrix components to create a suspension;

placing said suspension in a vessel having a three dimensional geometry approximating the *in vivo* gross morphology of said tissue, said vessel having attachment surfaces thereon;

allowing said suspension to coalesce; and

culturing said cells under conditions in which said cells form an organized tissue connected to said attachment surfaces.

22. The method of claim 21, wherein the step of providing comprises isolating primary cells of at least one of the cell types comprising said tissue.

23. The method of claim 21, wherein the step of providing comprises utilizing immortalized cells of at least one of the cell types comprising said tissue.

24. The method of claim 21, wherein the step of providing comprises inserting a foreign DNA sequence into at least one of the cells comprising said tissue.

25. The method of claim 24, wherein said tissue comprises substantially post-mitotic cells.

26. The method of claim 21, wherein said coalesced suspension exerts a force on said cells substantially parallel to a dimension of said vessel.

27. The method of claim 21, wherein said cells are aligned substantially parallel to a dimension of said vessel.

28. The method of claim 21, wherein said cells comprise skeletal muscle cells.

29. The method of claim 1, wherein said cells comprise myotubes.

30. The method of claim 27, wherein said vessel is substantially semi-cylindrical in shape.

31. The method of claim 30, wherein said attachment surfaces are positioned at opposite ends of said vessel.

32. The method of claim 21, wherein said tissue produces or mediates the production of a bioactive compound.

33. An organized tissue produced according to the method of claim 21.

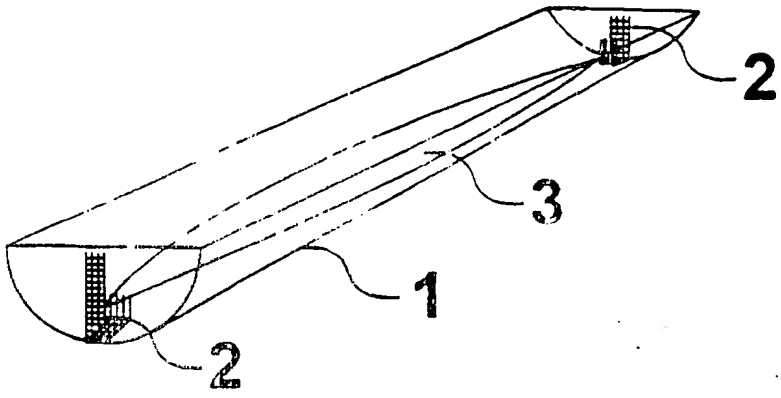
34. An apparatus for producing a tissue *in vitro* having an *in vivo*-like gross and cellular morphology, comprising a vessel having a three dimensional geometry approximating the *in vivo* morphology of said tissue; and tissue attachment surfaces in said vessel.

35. The apparatus of claim 34, further comprising a culture chamber in which said vessel may be submerged.

36. The apparatus of claim 34, wherein said vessel is substantially semi-cylindrical in shape.

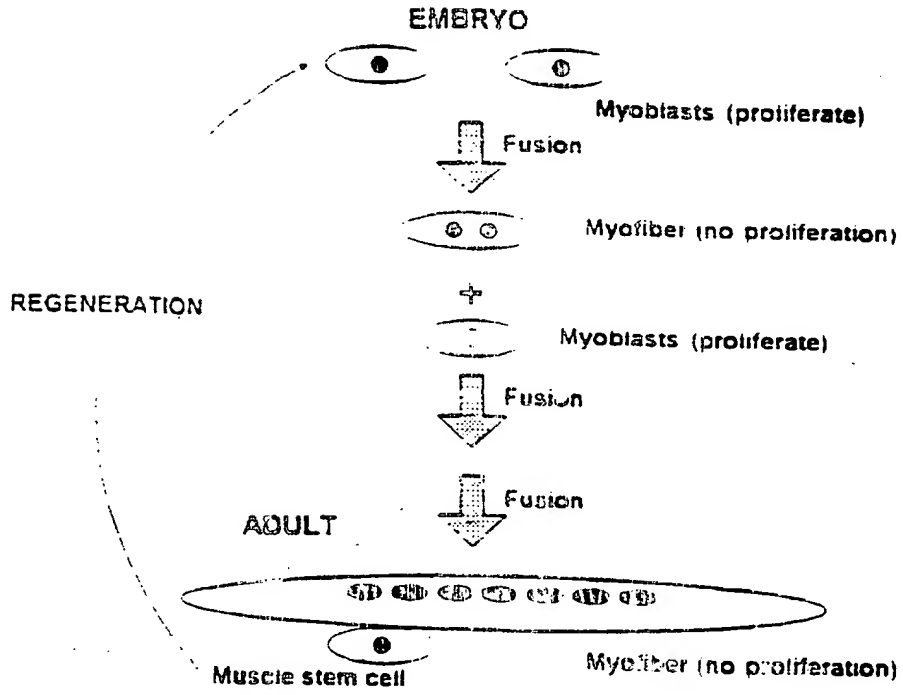
37. The apparatus of claim 36, wherein said tissue attachment surfaces are coupled to opposite ends of said semi-cylindrical vessel.

FIG. 1 Casting Chamber for Tissue Specimen Growth



patent2.vsd

FIG 2. SKELETAL MUSCLE GROWTH AND REGENERATION



patfig2b.vsd

FIGURE 3. RHGH SECRETING C2C12 CELL-MATRIX GEL 48 HOURS POSTPLATING. TOP GEL HAS DETACHED AND CONTRACTED.

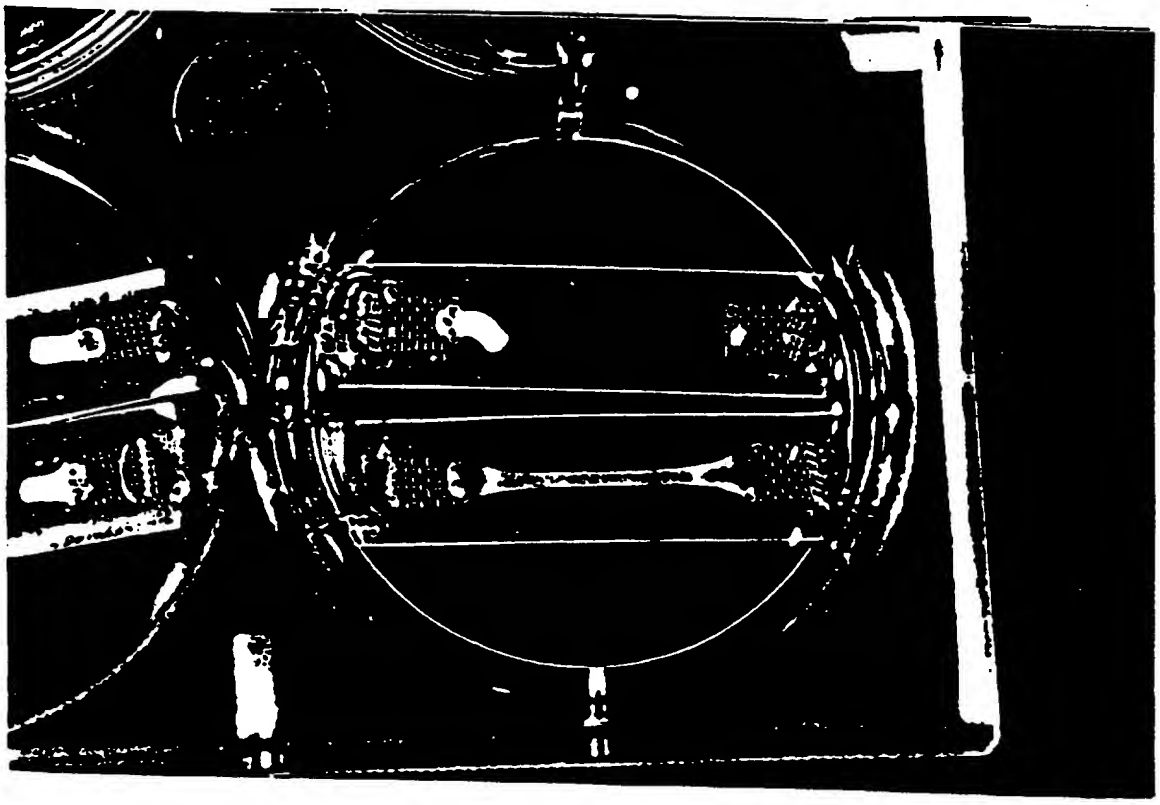


FIGURE 4. Middle section of a 3 weeks old mammalian C2C12 muscle cell organoid stained for sarcomeric tropomyosin, showing longitudinally oriented myofibers (arrows). Magnification is approximately 40X.

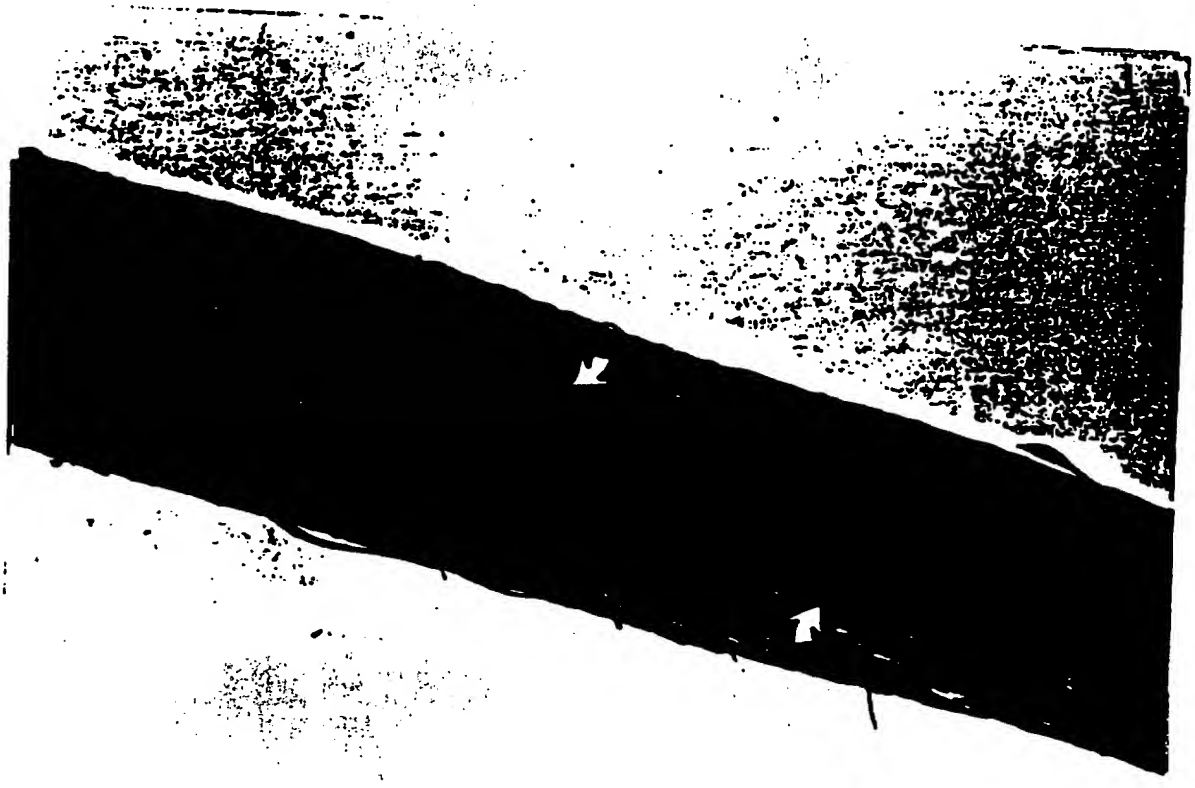


FIGURE 5. Parallel aligned myofibers (arrows) on the surface of a 3 weeks old mammalian C2C12 muscle cell organoid stained for sarcomeric tropomyosin. Magnification is approximately 400x.

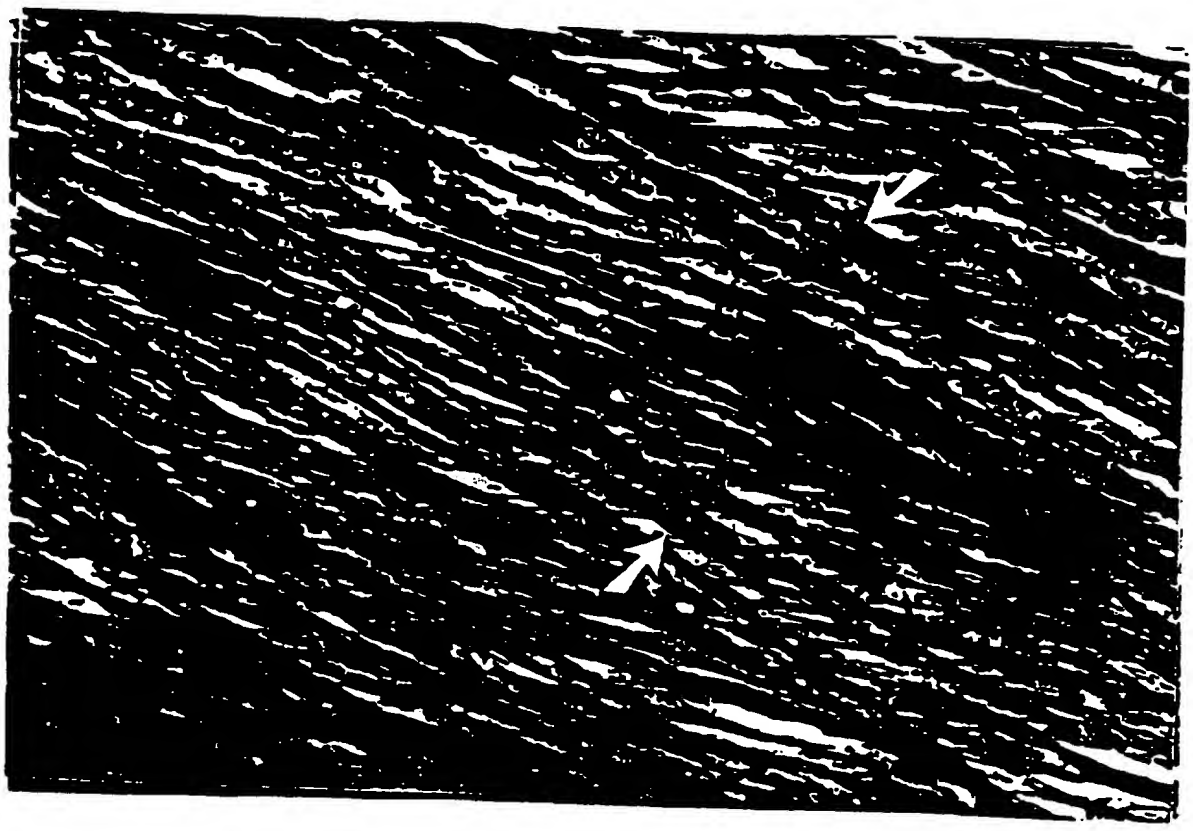
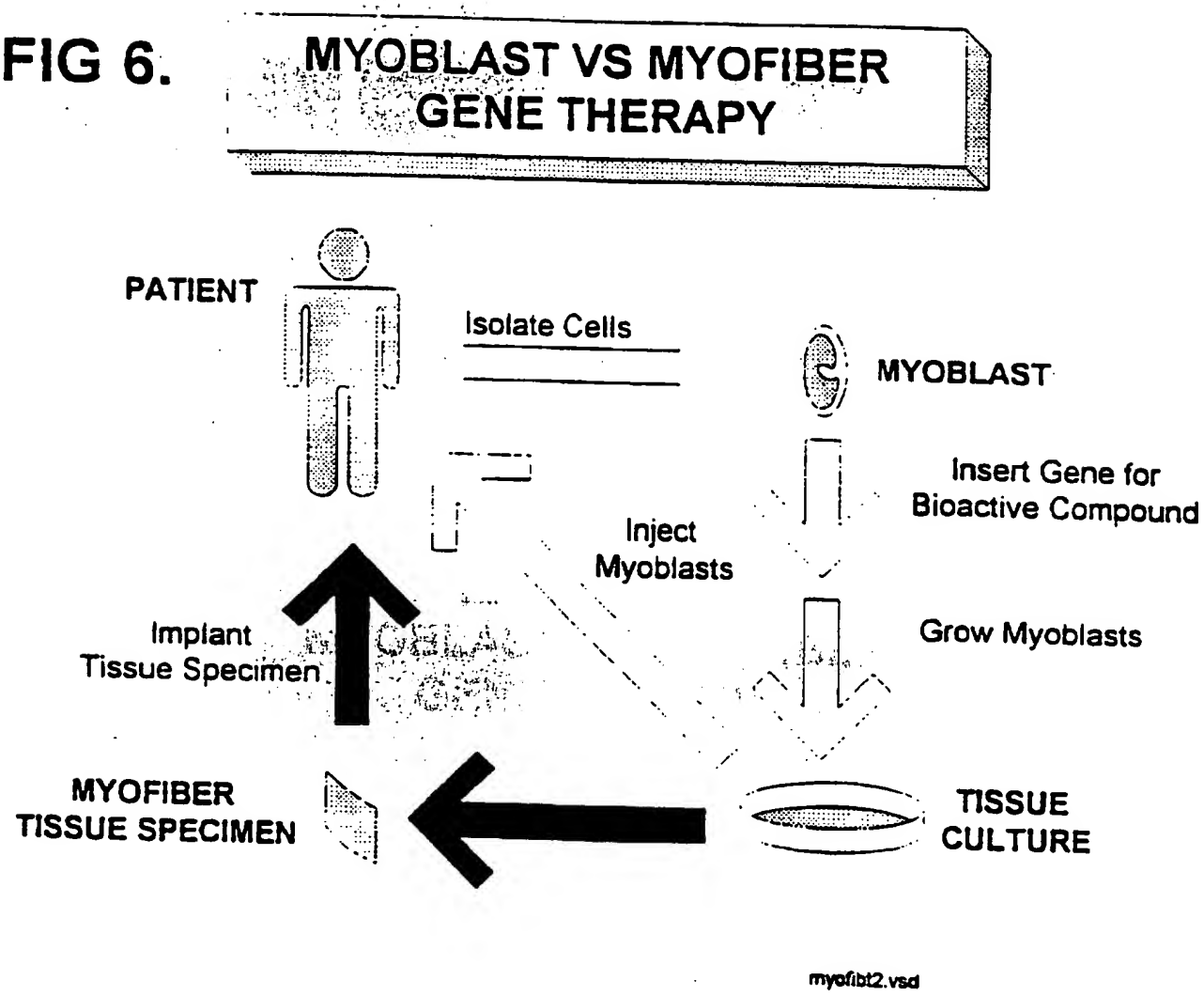


FIG 6.


MYOBLAST VS MYOFIBER GENE THERAPY
PATIENT
Isolate Cells
MYOBLAST
Insert Gene for Bioactive Compound
Inject Myoblasts
Implant Tissue Specimen
Grow Myoblasts
MYOFIBER TISSUE SPECIMEN
TISSUE CULTURE
myofibt2.vsd

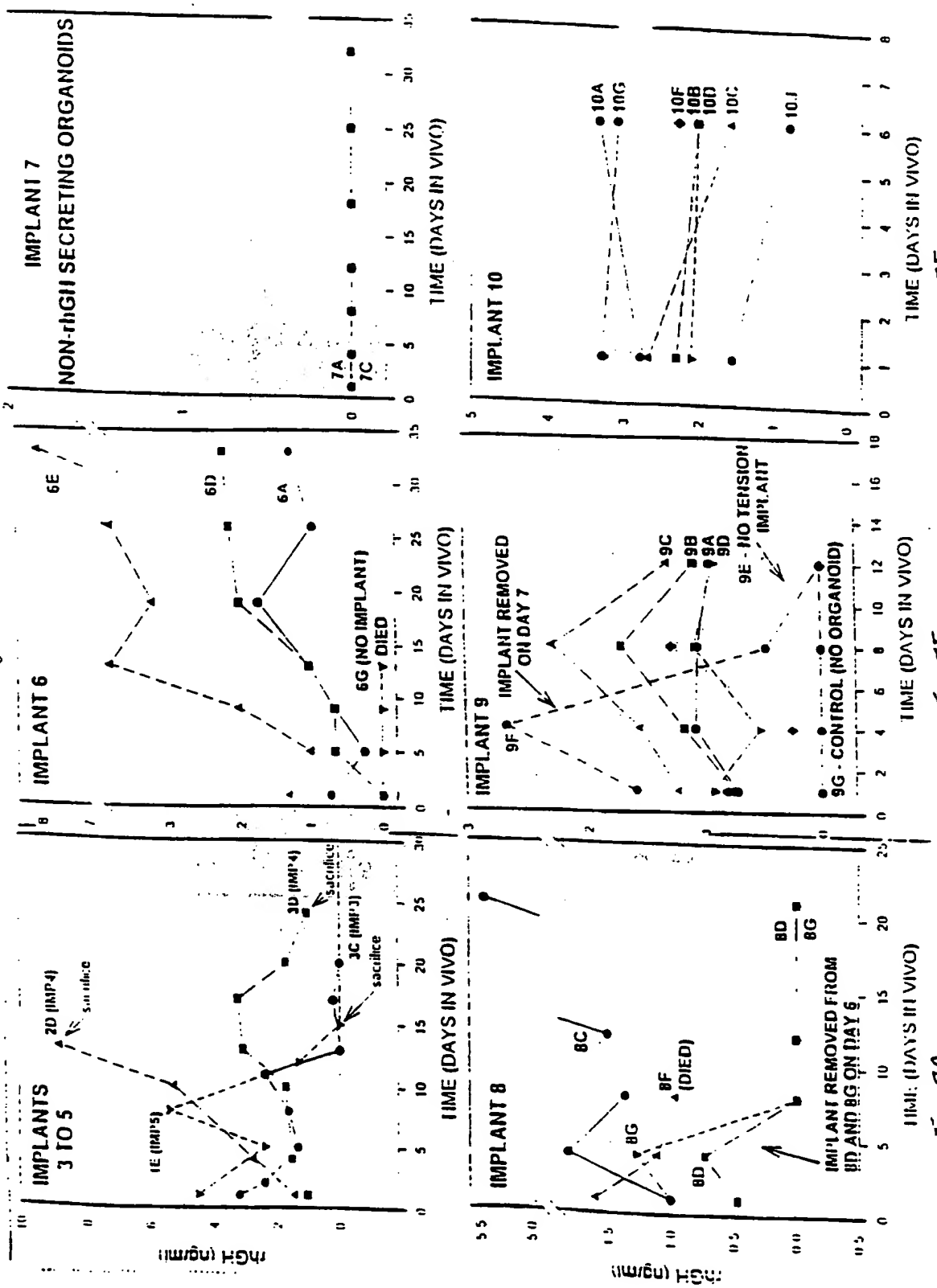


FIGURE 7A
IMPLANTS 3 TO 5
2D (IMP4)
3D (IMP4)
3C (IMP3)
sacrifice
hGH (ng/ml)
TIME (DAYS IN VIVO)
IMPLANT 6
6G (NO IMPLANT)
DIED
6D
6A
6E
IMPLANT 7
NON-rhGH SECRETING ORGANOIDS
7A
7C
IMPLANT 8
8C
8F (DIED)
8G
8D
IMPLANT REMOVED FROM 8D AND 8G ON DAY 6
8D
8G
IMPLANT 9
9F
IMPLANT REMOVED ON DAY 7
9C
9B
9A
9D
9E - NO TENSION IMPLANT
9G - CONTROL (NO ORGANOID)
IMPLANT 10
10A
10G
10F
10B
10D
10C
10J
Fig. 7B
Fig. 7C
Fig. 7D
Fig. 7E
Fig. 7F

FIGURE 8. Cytosine arabinoside (1µg/ml) for 4 days kills greater than 99% of proliferating C2C12 myoblasts (left graph) but has little effect on postmitotic myofibers (right graph).

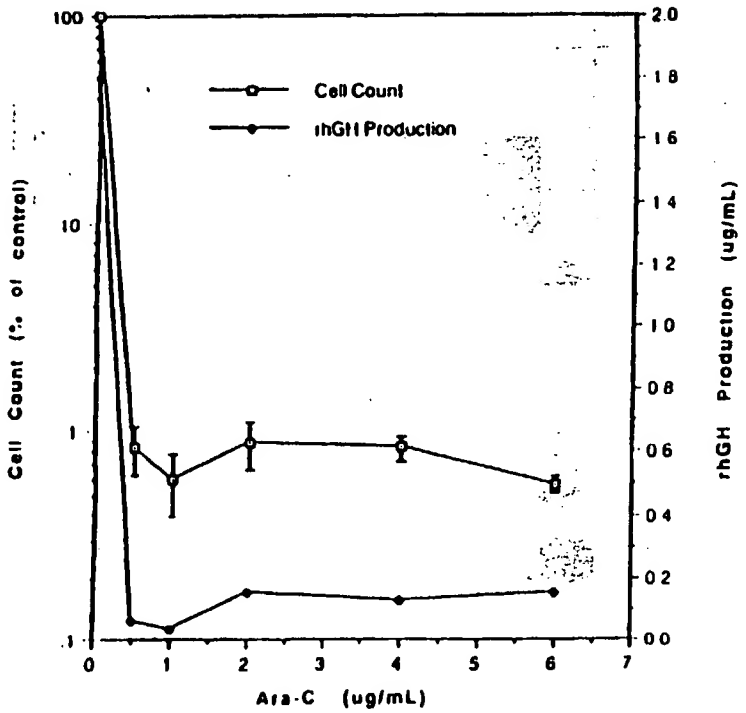


Fig. 8A

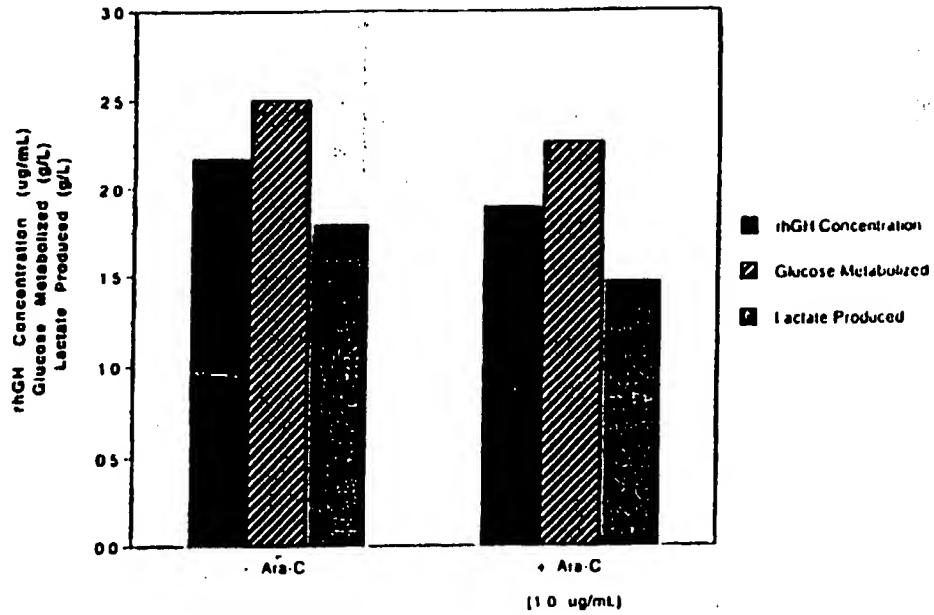


Fig. 8B

FIGURE 9. (A) rhGH secreting muscle organoid removed after 2 weeks in mouse 2D; (B) H & E stained cryostat cross-section of organoid shown in (A), with well differentiated myofibers running longitudinally in the organoid, and parallel to each other (arrows); and (C) X-gal blue staining of β-galactosidase activity in the cells containing the rhGH gene and β-gal gene (co-transfected in the same C2C12 myoblasts).

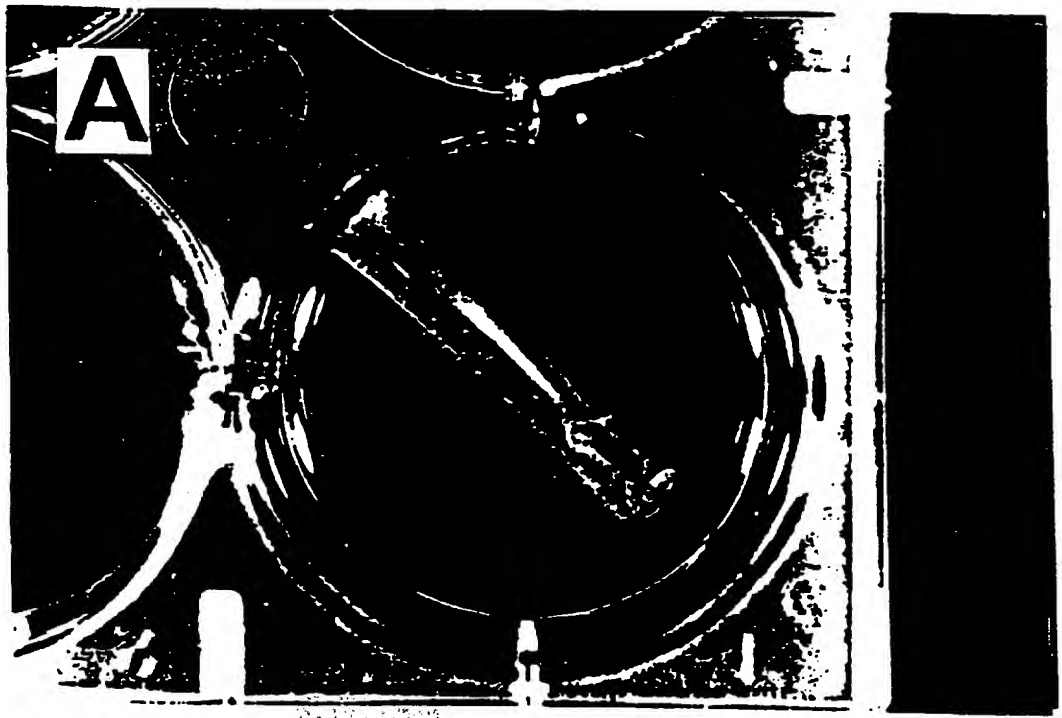
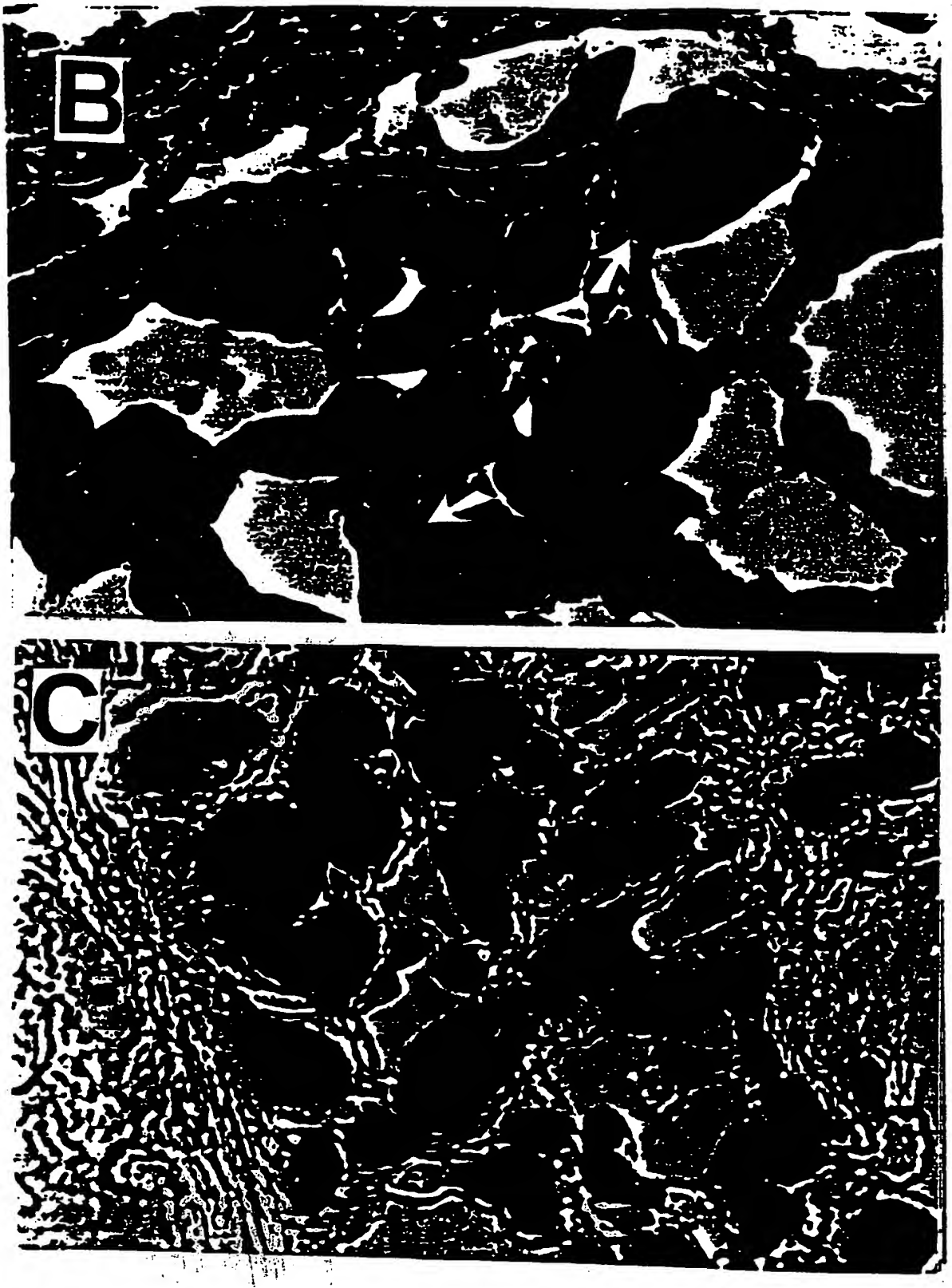


FIGURE 9. (con't)



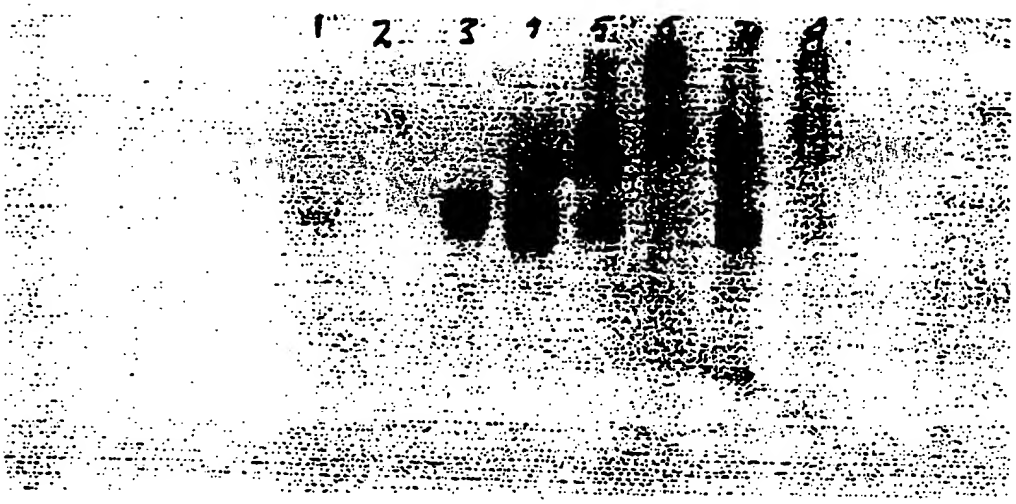


Figure 10A

Figure 10B

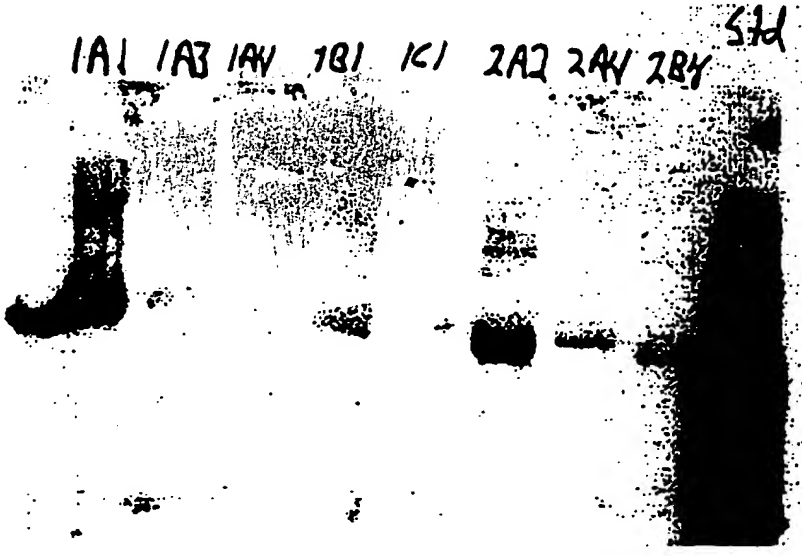
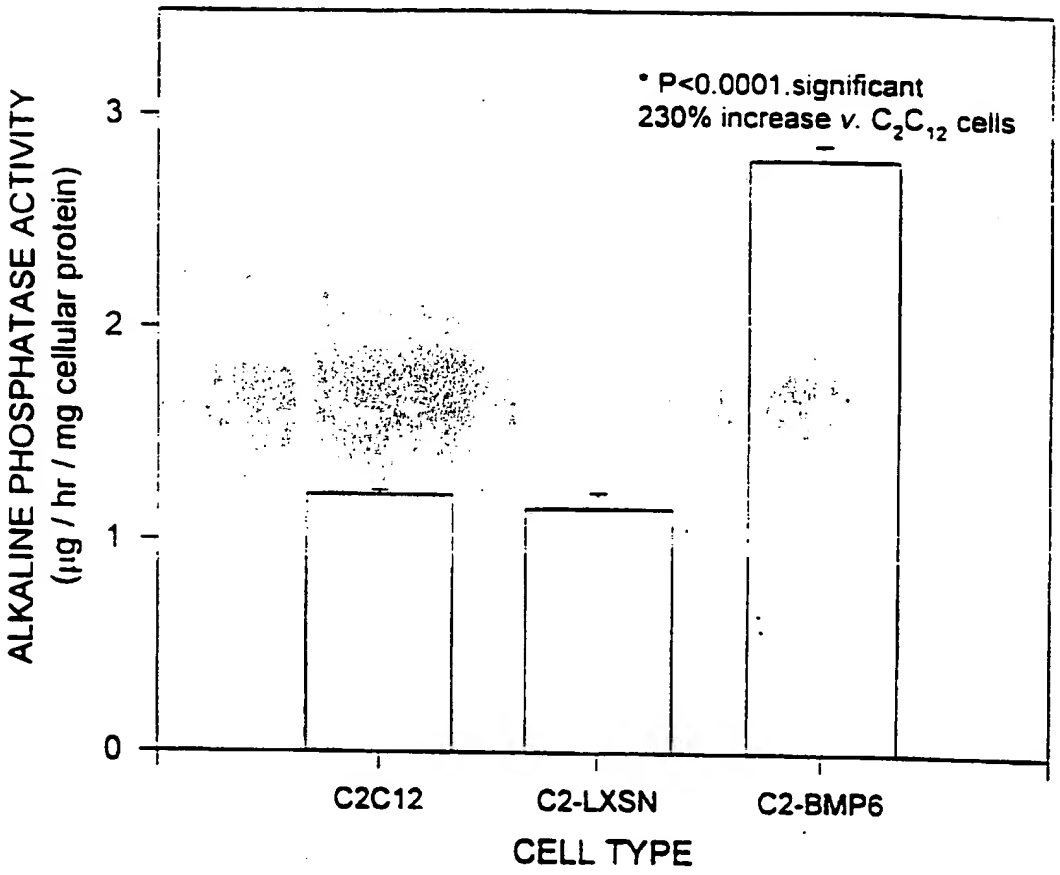


Figure 10C



Cell Type	n	Alkaline Phosphatase Activity (µg/h/mg cellular protein) (mean ± st. err.)	Total Cellular Protein (mg) (mean ± st. err.)
C_2C_{12} cells	4	1.21 ± 0.02	0.391 ± 0.019
C_2-LXSN cells	4	1.15 ± 0.07	0.413 ± 0.018
C_2-BMP6 cells	4	2.79 ± 0.07	0.381 ± 0.016

Fig. 11

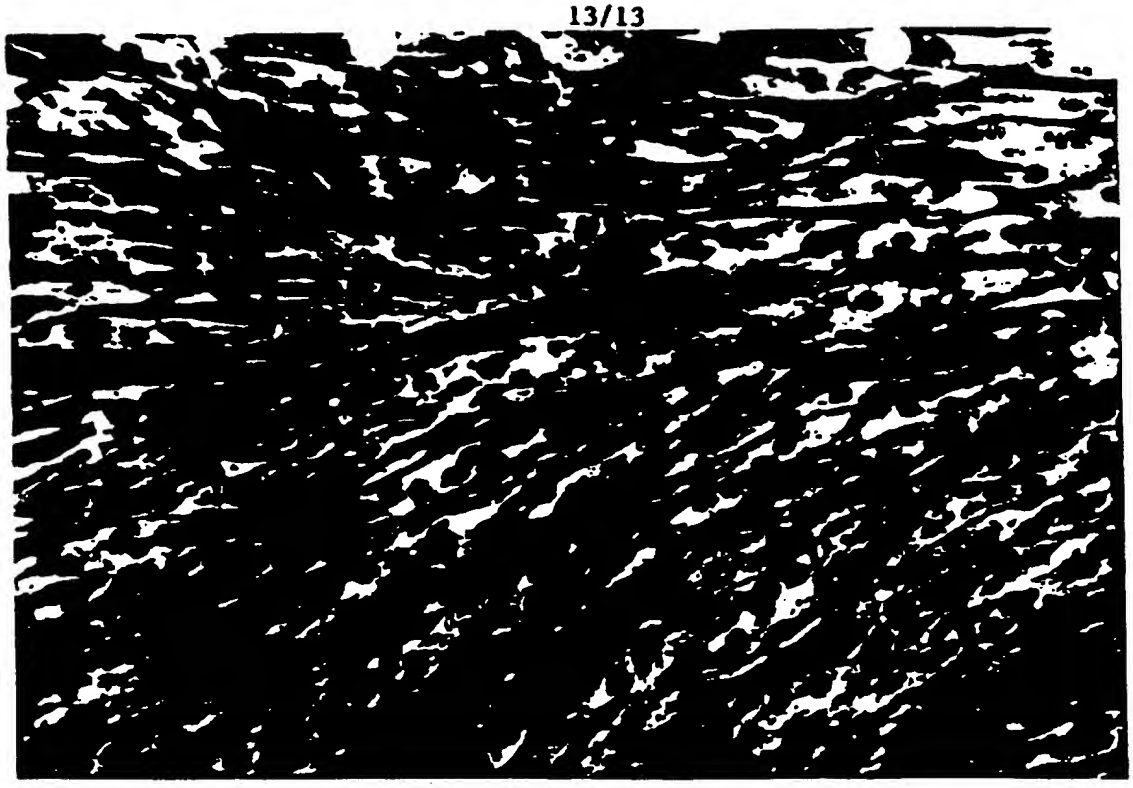


Fig. 12A

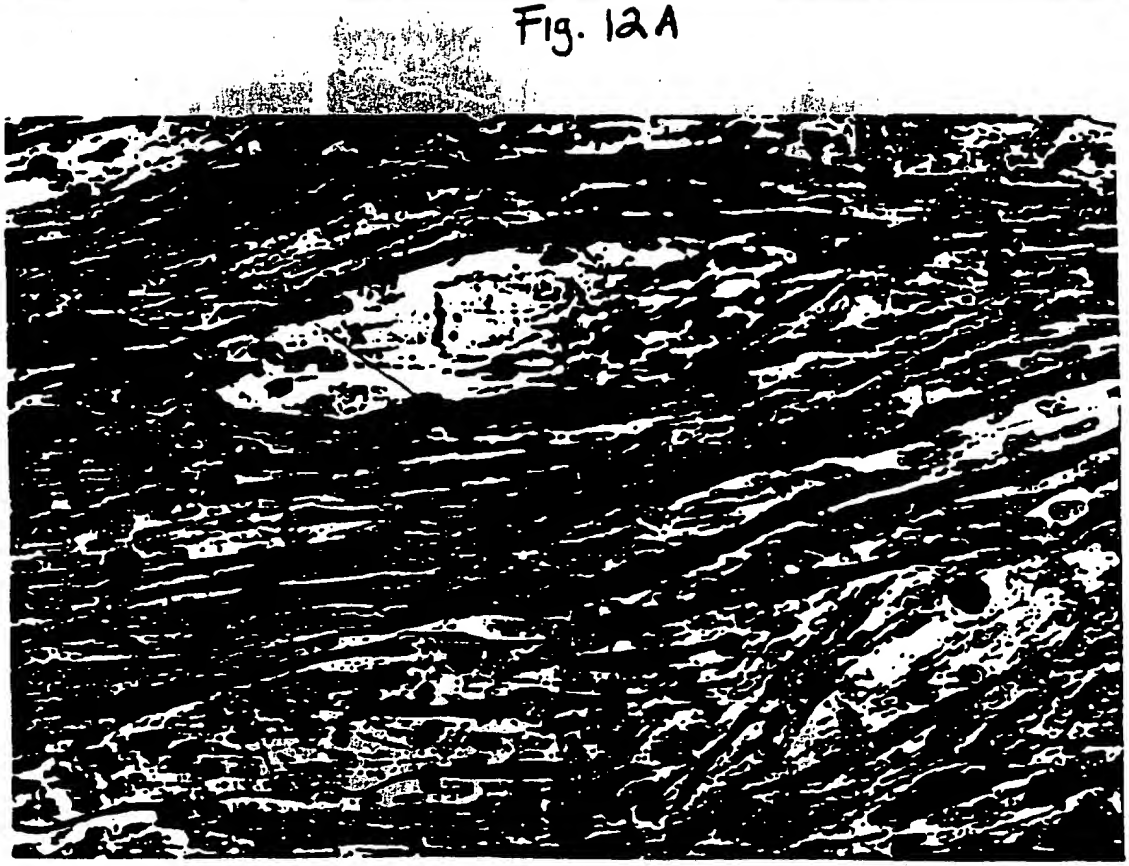


Fig. 12B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/00303

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/16 A61K48/00 C12M3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 00439 A (UNIV BROWN RES FOUND) 7 January 1993 see the whole document ---	1-37
X	WO 93 21859 A (GIAMPAPA VINCENT CARMINE) 11 November 1993 see the whole document ---	1,19
X	US 4 940 853 A (VANDENBURGH HERMAN H) 10 July 1990 see the whole document --- -/--	18-37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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1

Date of the actual completion of the international search	Date of mailing of the international search report
6 May 1997	27.05.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hillenbrand, G

Form PCT ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 97/00303

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 5, 1995, page 2099-2106 XP000652068 PERRONE, C.E. ET AL.: "Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-like growth factor binding proteins from differentiated skeletal muscle cells" see abstract ---	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/00303

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9300439 A	07-01-93	US 5182111 A	26-01-93
		AU 652071 B	11-08-94
		AU 2296192 A	25-01-93
		CA 2090719 A,C	29-12-92
		EP 0547203 A	23-06-93
		JP 6502080 T	10-03-94
WO 9321859 A	11-11-93	US 5326568 A	05-07-94
		AU 4370593 A	29-11-93
		CA 2134680 A	11-11-93
		EP 0637946 A	15-02-95
		JP 8500088 T	09-01-96
		US 5494677 A	27-02-96
		US 5580569 A	03-12-96
US 4940853 A	10-07-90	US 5153136 A	06-10-92